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# Increasing the thermal stability of the water-soluble pyrroloquinoline quinone glucose dehydrogenase by single amino acid replacement

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## Abstract

Based on the characterization of a PCR mutation of water-soluble glucose dehydrogenase possessing pyrroloquinoline quinone (PQQ), PQQGDH-B, Ser231Cys, we have constructed a series of Ser231 variants. The replacement of Ser231 to Cys, Met, Leu, Asp, Asn, His, or Lys resulted in an increase in thermal stability. Among these variants, Ser231Lys showed the highest level of thermal stability and also showed high catalytic activity. Considering that Ser231Lys showed more than an 8-fold increase in its half-life during the thermal inactivation at 55°C compared with the wild-type enzyme, and also retained catalytic activity similar to a wild-type enzyme, the application of this mutant enzyme as a glucose sensor constituent may develop into a stable glucose sensor construction. © 2000 Elsevier Science Inc. All rights reserved.

**Keywords:** Pyrroloquinoline quinone; Glucose dehydrogenase; Biosensor; Thermal stability

## 1. Introduction

Various bacterial enzymes were reported to possess pyrroloquinoline quinone (PQQ) as their prosthetic group, such as ethanol dehydrogenases (EDHs), methanol dehydrogenases (MDHs), and glucose dehydrogenases (GDHs). Among these PQQ enzymes, extensive attention has been given to PQQGDHs as enzyme sensor constituents [1–7], because of their property of being oxygen independent [7,8]. Two types of PQQGDHs have been reported; the membrane binding single peptide PQQGDH (PQQGDH-A), and the water soluble dimeric PQQGDH (PQQGDH-B). PQQGDH-A has been found in various Gram-negative bacteria. Despite their highly homologous primary structures, enzymatic characteristics, such as co-factor binding stability, thermal stability and substrate specificity are dependent upon the derived bacterial sources. Focusing on the high homology within PQQGDH-A, the authors have been attempting to identify the protein region responsible for each enzymatic characteristic using extensive homology analyses

together with mutational analyses [9–17]. On the contrary, PQQGDH-B, has only been reported to be found in the periplasmic space in the genus *Acinetobacter*. The primary structure of this enzyme was unique and had little homology with other PQQ enzymes. Homologous sequences were found in putative open reading frames in the genome of a cyanobacterium, *Synechocystis* spp. PCC 6803 [18] and in the intergenic region of *Escherichia coli* MOEA-DACC [19]. Although extensive studies have been carried out for the elucidation of the enzyme kinetics of PQQGDH-B [20–22], no structural information is available and no mutational analysis has ever been reported, though they are essential for the improvement of the enzymatic properties of PQQGDH using the protein engineering approach.

Here we report a site-directed mutagenesis study of PQQGDH-B based on a point mutant enzyme constructed by PCR amplification of the PQQGDH-B structural gene in this study. The mutant enzyme found in our laboratory possesses an amino acid substitution in the 231st position, Ser to Cys. This mutant, Ser231Cys showed higher thermal stability than the wild-type PQQGDH-B. Therefore, we replaced Ser231 with a series of amino acids, and analyzed their impact on thermal stability.

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Table 1  
Strains and plasmids

Strains and plasmids	Genotype or phenotype	Reference or source
<i>Escherichia coli</i>		
PP2418	ptsI, thi, gal P, gcd :: cat	Cleton-Jansen et al., 1990
BMH71-18 mutS	$\Delta(lac-proAB), thi, supE, mutS215::Tn10(tet^r)/F'$ <i>traD36, proAB, lacI<sup>q</sup>, lacZ<math>\Delta</math>M15</i>	Takara
MV1184	$\Delta(lac-proAB), ara, strA, thi, (\phi80lacZ\Delta M15), \Delta(srl-recA)$ $306::Tn10(tet^r)/F', traD36, proAB, lacI^q, lacZ\Delta M15$	Takara
Plasmids		
pKF18k	Km <sup>r</sup> ; <i>lacZ</i>	Takara
pGB	Ap <sup>r</sup> ; pTrc99A derivative carrying gene for NcoI-HindIII fragment of the <i>gdhB</i> gene (wild type)	This study

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* PP2418, of which the PQQGDH structural gene was disrupted by insertion mutagenesis [23], was used as the host strain for the expression of each PQQGDH-B. *Acinetobacter calcoaceticus* LMD79.41 was obtained from the Netherlands Culture Collection (NCC), as a source of the genomic fragment containing the PQQGDH-B structural gene. *E. coli* BMH71-18mutS and *E. coli* MV1184 were used for constructing mutations by site-directed mutagenesis. All the PQQGDH structural genes were inserted into the multi cloning site of the expression vector, pTrc99A (Pharmacia, Sweden).

### 2.2. Genetic manipulation

PCR amplification of the PQQGDH-B structural gene was carried out using a genomic gene extracted from *A. calcoaceticus* LMD79.41 as the template, and following one set of oligonucleotide as the primers.

Forward: 5'-GGCCATGGATAAACATTTATTG  
GCTAAAATTGCTTTAT-3'  
Reverse: 5'-GGAAGCTTTTACTTAGCCTTATAG  
GTGAACCTTAATGAG-3'

On each primer, a restriction enzyme site was designed, a *Nco*I site for forward primer and a *Hind*III site for a reverse primer, respectively. The introduction of *Nco*I site in the forward primer resulted in the substitution of the second amino acid residue in the signal peptide (Asn to Asp). The reaction was performed in a Perkin-Elmer's apparatus under the conditions described by Saiki et al. [24], using as enzyme the Taq DNA polymerase as the enzyme (Takara, Japan). Thus amplified fragment was digested by *Nco*I-*Hind*III, and inserted in the multi-cloning site of pTrc99A. The sequence of inserted gene fragments was analyzed with an automated DNA sequencer (Perkin Elmer ABI Model 310), and the clone harboring identical sequence

with the wild-type PQQGDH-B gene except the PCR primer region, was designated as pGB (Fig. 1).

Site-directed mutagenesis was carried out using the gene fragment (1.2 kbp) obtained by *Kpn*I-*Hind*III digestion from the aforementioned PQQGDH-B structural gene inserted in pTrc99A. The resultant fragment was inserted in the linearized pKF18k (Takara, Japan), after digestion by *Kpn*I and *Hind*III. The oligonucleotide primers used for the site-directed mutagenesis are summarized in Fig. 2.

Site-directed mutagenesis was performed according to the instruction manual. The nucleotide sequence of mutation was confirmed by an automated DNA sequencer (Perkin-Elmer ABI Model 310). This obtained mutated region was then digested with *Kpn*I and *Hind*III, and the fragment was substituted into the corresponding region of pGB. In this way, expression vectors containing mutated PQQGDH-B were constructed.

### 2.3. Enzyme preparation

The expression vector containing a wild type or each mutated PQQGDH-B structural gene was transformed into *E. coli* PP2418, and cultivated according to the previous study [25], except for the addition of 1 mM of CaCl<sub>2</sub> instead of MgCl<sub>2</sub>. The cells were harvested after the late log phase,

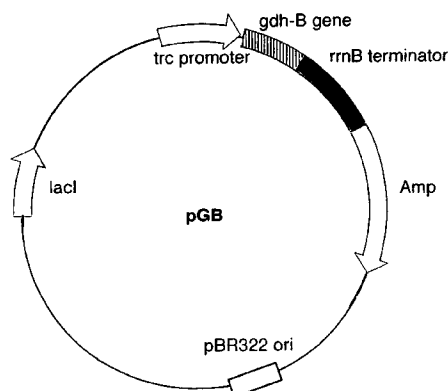


Fig. 1. The construction of plasmid pGB.

	225	226	227	228	229	230	231	232	233	234	235
Wild type (Ser231)	3'- CG	AAT	TTA	GAA	CTA	CCT	TCA	TAA	GGT	TTC	C -5'
	Arg	Leu	Asn	Leu	Asp	Gly	Ser	Ile	Pro	Lys	Asp
Ser231Cys	3'- CG	AAT	TTA	GAA	CTA	CCT	ACA	TAA	GGT	TTC	C -5'
							Cys				
Ser231Asp	3'- CG	AAT	TTA	GAA	CTA	CCT	CTA	TAA	GGT	TTC	C -5'
							Asp				
Ser231His	3'- CG	AAT	TTA	GAA	CTA	CCT	GTA	TAA	GGT	TTC	C -5'
							His				
Ser231Lys	3'- CG	AAT	TTA	GAA	CTA	CCT	TTT	TAA	GGT	TTC	C -5'
							Lys				
Ser231Leu	3'- CG	AAT	TTA	GAA	CTA	CCT	TAC	TAA	GGT	TTC	C -5'
							Leu				
Ser231Met	3'- CG	AAT	TTA	GAA	CTA	CCT	TGA	TAA	GGT	TTC	C -5'
							Met				
Ser231Asn	3'- CG	AAT	TTA	GAA	CTA	CCT	TTA	TAA	GGT	TTC	C -5'
							Asn				

Fig. 2. The oligonucleotide primers and corresponding peptide sequences of Ser231 variants of PQQGDH-B.

resuspended in 10 mM phosphate buffer pH 7.0 containing 5 mM  $MgCl_2$ , and disrupted by French Pressure (110 MPa). The sample was then subjected to ultra-centrifugation ( $160\,500 \times g$  1.5 h, 4°C), following the dialysis in 10 mM potassium phosphate buffer, pH 7.0. The obtained supernatant was used as the crude enzyme preparation. The crude enzyme preparation was applied to a CM-Toyopearl 650 M cation exchange column (Toso, Japan) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. After the column was washed with the same buffer (two column volumes), the enzyme was eluted with a linear gradient of 0 to 0.2 M NaCl in 10 mM potassium phosphate buffer, pH 7.0. The fractions showing enzyme activity were applied to a hydroxy apatite column (Merck, Germany), previously equilibrated with a 10 mM potassium phosphate buffer, pH 7.0 containing 0.2 M NaCl, and eluted with a linear gradient of 0.2 to 0.5 M NaCl in a 10 mM potassium phosphate buffer, pH 7.0. The purified enzyme, found to be electrophoretically homogeneous by silver staining on SDS-PAGE, was utilized for kinetic studies.

#### 2.4. Analysis of thermal stability

Crude enzyme samples [protein concentration; 0.72 mg/ml (Wild type), 2.19 mg/ml (Ser231Met), 0.92 mg/ml (Ser231His), 7.94 mg/ml (Ser231Leu), 1.43 mg/ml (Ser231Asp), 1.14 mg/ml (Ser231Cys), 9.82 mg/ml (Ser231Asn), 2.65 mg/ml (Ser231Lys)] for the experiments shown in Fig. 3 and Table 1, or purified Ser231Lys sample for the experiment shown in Fig. 4A and B were subjected for the following experiments. The time courses of thermal inactivation were measured as follows. Each enzyme sample was incubated in a 10 mM MOPS buffer, pH 7.0 with 1 mM  $CaCl_2$  and 1  $\mu$ M PQQ for an hour at room temperature and then subjected to thermal inactivation experiments. The thermal inactivation was measured by incubating 183  $\mu$ l of an enzyme solution in 10 mM MOPS buffer, pH 7.0, at 55°C. Samples were taken every 2 or 10 min. Then, samples were stored at 4°C for 2 min and subsequently incubated at room temperature for 30 min. The residual enzyme activity

was determined in the presence of 0.6 mM phenazine methosulfate, 0.06 mM 2,6-dichlorophenolindophenol (DCIP) at 25°C, and a 100 mM glucose concentration, by measuring the decrease in absorption of DCIP at 600 nm.

The thermal stability of Ser231Lys at various temperature was determined by incubated purified enzyme sample at each temperature for 10 min. The residual enzyme activities were determined as shown above and were compared with the initial activity.

#### 2.5. Analysis of substrate specificity

Wild-type and mutant PQQGDH-Bs activities toward each substrate were obtained at 20 mM, and were compared with the activity for 20 mM glucose as a control, using crude enzyme preparations as enzyme samples. Kinetic parameters were determined by using a purified enzyme. Enzyme samples were incubated in a 10 mM MOPS buffer, pH 7.0, containing 1 mM  $CaCl_2$  and 1  $\mu$ M PQQ to form a holo

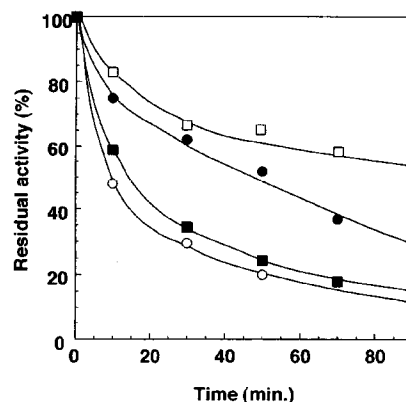


Fig. 3. Time courses of thermal inactivation of representative Ser231 variants of PQQGDH-B at 55°C. ○, Wild-type (0.72 mg/ml); ●, Ser231Cys (1.14 mg/ml); □, Ser231Lys (2.65 mg/ml); ■, Ser231Leu (7.94 mg/ml). Samples were taken every 2 or 10 min, stored at 4°C for 2 min, and subsequently incubated at room temperature for 30 min. The residual activity of PQQGDHs was determined at 25°C.

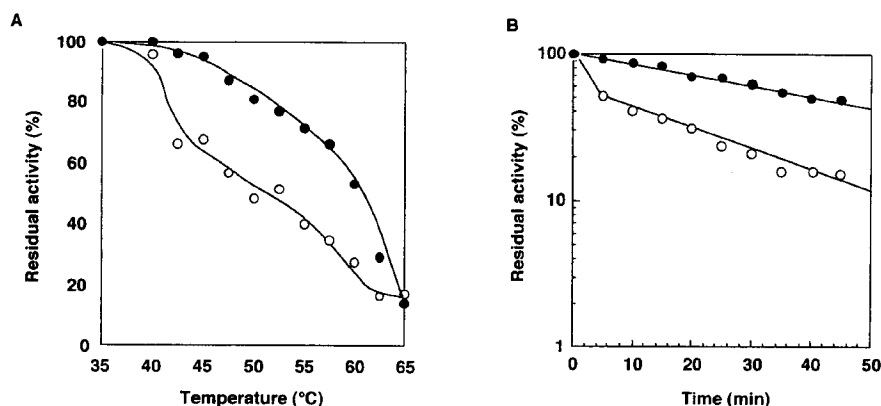


Fig. 4. Thermal stability of wild type and Ser231Lys PQQGDH-Bs. A, residual activity was determined at 25°C, after 10 min of incubation at each temperature. Protein concentration: wild type, 0.07  $\mu\text{g/ml}$ ; Ser231Lys, 0.2  $\mu\text{g/ml}$ . B, time course of thermal inactivation at 55°C was measured by taking each sample every 2 or 10 min. Other experimental condition were the same as in Fig. 3. Protein concentration: wild type, 0.07  $\mu\text{g/ml}$ ; Ser231Lys, 0.2  $\mu\text{g/ml}$ . O, Wild-type; ●, Ser231Lys

enzyme. Enzyme activity was measured as above, except for the utilization of an adequate concentration of each substrate.

### 3. Results and discussion

#### 3.1. The impact of the Ser231 replacement

Fig. 3 shows the time courses for the thermal inactivation of representative Ser231 variants of PQQGDH-B at 55°C. Because the residual enzyme activity was measured after cooling process and also incubation at room temperature, these experiments demonstrated the time courses of irreversible thermal inactivation. The Ser231Cys was constructed during the PCR amplification of the *A. calcoaceticus* LMD79.41 gene in this study. Ser231Cys showed much higher thermal stability than the wild-type PQQGDH-B. Based on this finding, we constructed six more variants in the 231st position of PQQGDH-B; Ser231Met, Ser231His, Ser231Asp, Ser231Asn, Ser231Leu, and Ser231Lys. All the

variants showed similar or higher thermal stability than the wild-type enzyme. The residual activity of wild type enzyme after 30 min of incubation at 55°C was 30%, and Ser231His (31%), Ser231Leu (34%), Ser231Met (36%), and Ser231Asp (44%) showed similar or slightly higher thermal stability. In contrast, Ser231Cys (62%), Ser231Asn(64%), and Ser231Lys (66%) showed more than 60% of residual activity, and showed increased thermal stability. Therefore, these replacements resulted in an increase in the thermal stability of PQQGDH-B.

Table 2 summarizes the substrate specificity of Ser231 variants of PQQGDH-B, including the wild-type recombinant PQQGDH-B prepared in this study. PQQGDH-B is famous for its wide substrate specificity, and is differentiated from PQQGDH-A by oxidizing disaccharides such as lactose, but not 2-deoxy-glucose [26]. All of the mutations constructed in this study showed similar substrate specificity to the wild type, on the basis of the above mentioned characteristics against PQQGDH-A. However, some of the mutants showed different substrate specificity profiles from that of wild type one. Ser231Leu and Ser231Asn showed

Table 2  
Substrate specificity of Ser231 variants

	Ser231(Wild type)	Ser231Lys	Ser231Cys	Ser231Asp	Ser231Leu	Ser231Asn	Ser231Met	Ser231His
Glucose	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)	100(%)
2-deoxy-D-glucose	4	5	3	2	6	5	5	2
Mannose	13	10	8	9	13	12	9	12
Allose	47	43	46	38	62	61	43	57
3-O-methyl-D-glucose	81	82	76	71	105	109	80	86
Galactose	11	15	14	12	20	18	10	17
Xylose	7	5	8	8	12	15	8	7
Lactose	61	59	69	54	73	66	56	56
Maltose	61	70	69	38	76	51	41	38

Each enzyme activity was measured at 20 mM substrate concentration.

The values were the relative activity compared with the activity toward glucose as the substrate.

higher activity toward allose (Ser231Leu; 62%, Ser231Asn; 61%), 3-*O*-methyl-D-glucose (Ser231Leu; 105%, Ser231Asn; 109%), galactose (Ser231Leu; 20%, Ser231Asn; 18%), and toward xylose (Ser231Leu; 12%, Ser231Asn; 15%), than wild type (allose; 47%, 3-*O*-methyl-D-glucose; 81%, galactose; 11%, and xylose; 7%). Ser231His also showed increased activity toward galactose (17%). In contrary, Ser231Asp and Ser231Met showed decreased activity toward maltose (Ser231Asp; 38%, Ser231Met; 41%, wild type; 61%). Ser231Lys and Ser231Cys showed almost identical substrate specificity with wild type one. Because only a slight alteration in the substrate specificity was observed by the substitution at Ser231 residue, this residue may not govern the substrate specificity of this enzyme.

Considering that the hydrophobicity of the amino acid residues substituted in this study, neither thermal stability nor substrate specificity has correlation with the hydrophobicity at Ser231 residue. It is also obvious, the charge or the size of side chain of 231 residue did not show any correlation with thermal stability or with substrate specificity. For the precise interpretation of the observed alteration in the enzymatic characteristics, the information about the properties of substituted amino acid residues is not sufficient, but further structural information of PQQGDH-B will be needed. Considering our preliminary circular dichroic (CD) study together with secondary structure motif prediction (results not shown) revealed that PQQGDH-B is a  $\beta$ -propeller protein composed of 6-W motifs, as another PQQ enzymes structural motifs. Because the alteration of Ser231 residue affected on both thermal stability and on substrate specificity, this residue may have a significant role in maintaining  $\beta$ -propeller structural motif.

Considering that Ser231Lys showed the highest thermal stability, further detailed characteristics of this mutant enzyme was carried out using a purified enzyme sample.

### 3.2. The enzymatic properties of Ser231Lys

The thermal stability of Ser231Lys was investigated. Fig. 4A shows the residual activity of Ser231Lys and wild type PQQGDH-B after 10 min of incubation at each temperature. Over the range tested, Ser231Lys showed higher thermal stability than the wild type. Fig. 4B shows the time course on thermal inactivation at 55°C. The wild type purified enzyme sample rapidly inactivated at this temperature and within 5 min of incubation, the enzyme showed about 50% of the initial activity. In contrast, Ser231Lys inactivated much slower than wild type. Considering that the time course of thermal inactivation was well describable by first-order kinetics, from the linear regression of logarithmic of residual activity against time, the half-life time of Ser231Lys at 55°C was calculated as 41 min, which was longer than 8-fold compared with the wild type one.

The kinetic parameters of Ser231Lys and wild type PQQGDH-B are summarized in Table 3. As reported previously [27], substrate inhibition was observed at a glucose

Table 3  
Kinetic parameters for glucose of PQQGDH-B

	K <sub>m</sub> (mM)	V <sub>max</sub> (U/mg protein)	Specific activity <sup>a</sup> (U/mg protein)
Wild type	25	3821	2977 <sup>b</sup>
Ser231Lys	27	4985	3313 <sup>c</sup>

One unit enzyme activity corresponds to the conversion of 1  $\mu$ mol DCIP per minute under the assay condition.

<sup>a</sup> The highest activity observed in each sample was expressed as the specific activity.

<sup>b</sup> Enzymatic activity observed at 50 mM glucose concentration.

<sup>c</sup> Enzymatic activity observed at 100 mM glucose concentration.

concentration higher than 100 mM. The specific activity (the highest enzyme activity observed) of Ser231Lys was 3313 U/mg. On the basis of Michaelis–Menten equation, V<sub>max</sub> value and K<sub>m</sub> value for glucose was calculated as 4985 U/mg and 27 mM, respectively. These values closely resembled those of the wild type GDH-B, prepared in this study.

The specific activity of wild-type PQQGDH-B has been reported by several authors, and the highest activity so far recorded was 7400 U/mg [21]. However, other papers reported about 3000 U/mg (2214 U/mg by Matsushita et al., 1995 [20]; 4000 U/mg by Olsthoorn et al., 1998 [22]). Although the specific activity of Ser231Lys is less than the highest wild-type PQQGDH-B activity so far reported, it retains more than 3000 U/mg, more than 10-fold of the specific activity of glucose oxidase, which is commonly utilized as the glucose enzyme sensor. Because Ser231Lys showed increased thermal stability and also retained similar specific activity to wild-type enzyme, several advantages can be expected if this mutant enzyme is being applied for the glucose sensor constituent. Considering that Ser231Lys is much stable than wild type enzyme, higher yield in active enzyme preparation is expected, which may result in costly effective glucose sensor component production. Usually, the amount of enzyme is determined on the basis of remaining activity after whole process of enzyme sensor construction, considering the loss of the activity during the process. Therefore, the higher thermal stability of Ser231Lys than wild type enzyme may prevent the inactivation of enzyme during enzyme sensor production, consequently, allow the minimizing the amount of enzyme to be loaded on electrode. Moreover, the higher thermal stability usually results the higher storage stability, therefore, longer shelf-life of the sensor employing Ser231Lys is expected than that employing wild type enzyme. Therefore, the application of Ser231Lys may realize cost effective and also stable glucose enzyme sensor.

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